Nuclease

Week 15

Summarized below are the experiments conducted this week in chronological order. Click on the experiment name to view it. To go back to this summary, click **Summary** in the footer.

Summary

1 Addition of BamHI restriction site downstream to the nuclease sequence and amplification of the whole plasmid, using overhang PCR - third attempt

Responsible

Oscar He and Reskandi Rudjito

Protocols used

PCR Amplification (Phusion)

Modifications and comments to protocols

PCR Amplification (Phusion) primers:	Nuc_R (reverse) Suffix_F (forward)
PCR Amplification (Phusion) buffer:	GC Buffer
PCR Amplification (Phusion) extension time:	120 seconds

Experimental Set Up

Table 1: Concentration of samples used for the ovarhang PCR amplification.

Sample	Concentration [ng/μl]
pSB1C3-T7-Nuc (diluted from 288.6 ng/μl)	10

Table 2: Volumes of components in PCR mix.

Component	Volume [μl]
10 μM Suffix_F	1
10 μM Nuc_R	1
5X Phusion GC Buffer	4
Phusion DNA Polymerase	0.2
2 mM dNTP mix	2
Template DNA	1
ddH_2O	10.8

Table 3: Thermocycling conditions, with two different annealing temperatures.

Step	Temperature [°C]	Time [sec]
Initial Denaturation	98	30
30 x	98	10
	55 (57)	30
	72	120
Final Extension	72	600
Hold	4	N/A

Results and Conclusions

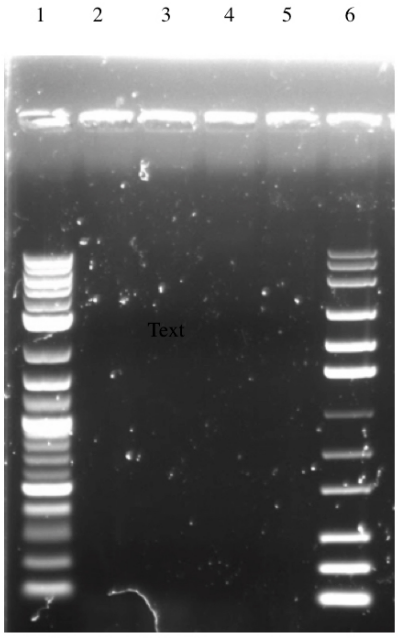

Figure 1: Overhang PCR of pSB1C3-T7-Nuc third attempt.(1) Ladder (2) Nuc annealed at 55 °C (3) Nuc annealed at 57 °C (4)-(5) Not of interest(6) Ladder

Discussion and Troubleshooting

The extension time of 120 seconds was probably too long, and the reason for the unsuccessful PCR. Another attempt will be performed with the previous extension time and instead a higher annealing temperature.

2 Addition of BamHI restriction site downstream to the nuclease sequence and amplification of the whole plasmid, using overhang PCR - fourth attempt

Responsible

Oscar He

Protocols used

PCR Amplification (Phusion)

Modifications and comments to protocols

PCR Amplification (Phusion) primers:	Nuc_R (reverse) Suffix_F (forward)
PCR Amplification (Phusion) buffer:	GC Buffer
PCR Amplification (Phusion) extension time:	75 seconds
PCR Amplification (Phusion) annealing temperature:	65 and 70 °C

Experimental Set Up

Table 4: Concentration of samples used for the ovarhang PCR amplification.

Sample	Concentration [ng/μl]
pSB1C3-T7-Nuc (diluted from 288.6 ng/μl)	10

Table 5: Volumes of components in PCR mix.

Component	Volume [μl]
10 μM Suffix_F	1
10 μM Nuc_R	1
5X Phusion GC Buffer	4
Phusion DNA Polymerase	0.2
2 mM dNTP mix	2
Template DNA	1
ddH_2O	10.8

Table 6: Thermocycling conditions, with two different annealing temperatures.

Step	Temperature [°C]	Time [sec]
Initial Denaturation	98	30
30 x	98	10
	65 (70)	30
	72	75
Final Extension	72	600
Hold	4	N/A

Results and Conclusions

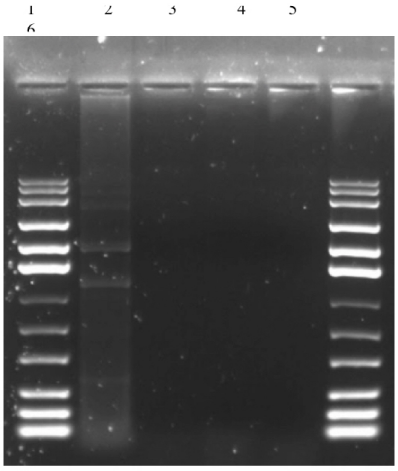

Figure 2: Overhang PCR of pSB1C3-T7-Nuc fourth attempt.(1) Ladder (2) Nuc annealed at 65 °C (3) Not of interest °C (4)Nuc annealed at 70 °C (5) Not of interest(6) Ladder

Discussion and Troubleshooting

The annealing temperature of 70 °C was set according to the NEB T_m Calculator for the used primers (actually 72 °C). This was probably too high since there were no bands on the gel, except for two faint bands for the nuclease construct at 65 °C, indicating failure of the primers to bind to the template. For further trials, the annealing temperature will be decreased and the extension time slightly increased. If the concentration of PCR product is still not increased compared to the concentration acquired in the second attempt, another attempt will be performed where only the number of cycles will be increased.

3 Addition of BamHI restriction site downstream to the nuclease sequence and amplification of the whole plasmid, using overhang PCR - fifth attempt

Responsible

Oscar He

Protocols used

PCR Amplification (Phusion)

Modifications and comments to protocols

PCR Amplification (Phusion) primers:	Nuc_R (reverse) Suffix_F (forward)
PCR Amplification (Phusion) buffer:	GC Buffer
PCR Amplification (Phusion) extension time:	90 seconds
PCR Amplification (Phusion) annealing temperature:	60 °C

Experimental Set Up

Table 7: Concentration of samples used for the ovarhang PCR amplification.

Sample	Concentration [ng/μl]
pSB1C3-T7-Nuc (diluted from 288.6 ng/μl)	10

Table 8: Volumes of components in PCR mix.

Component	Volume [μl]
10 μM Suffix_F	1
10 μM Nuc_R	1
5X Phusion GC Buffer	4
Phusion DNA Polymerase	0.2
2 mM dNTP mix	2
Template DNA	1
ddH_2O	10.8

Table 9: Thermocycling conditions, with two different annealing temperatures.

Step	Temperature [°C]	Time [sec]
Initial Denaturation	98	30
30 x	98	10
	60	30
	72	90
Final Extension	72	600
Hold	4	N/A

Results and Conclusions

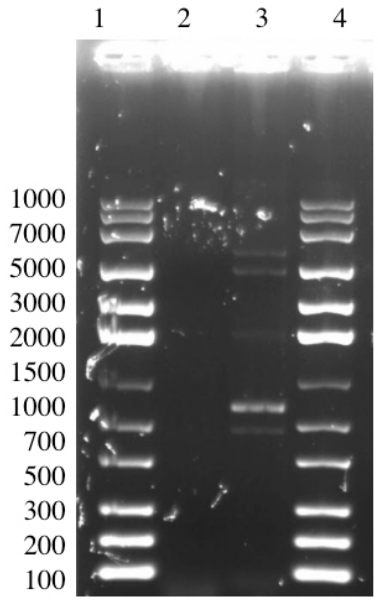

Figure 3: Overhang PCR of pSB1C3-T7-Nuc fifth attempt.(1) Ladder (2) Nuc annealed at 60 °C (3) Not of interest (4) Ladder

Discussion and Troubleshooting

The annealing temperature was probably too high for the nuclease construct. Another PCR amplification will be performed with the same conditions as in the second attempt but with 35 cycles instead of 30 in hope of increasing the concentration of the product.

4 Addition of BamHI restriction site downstream to the nuclease sequence and amplification of the whole plasmid, using overhang PCR - sixth attempt

Responsible

Oscar He

Protocols used

PCR Amplification (Phusion)

Modifications and comments to protocols

PCR Amplification (Phusion) primers:	Nuc_R (reverse) Suffix_F (forward)
PCR Amplification (Phusion) buffer:	GC Buffer
PCR Amplification (Phusion) extension time:	60 seconds
PCR Amplification (Phusion) annealing temperature:	57 °C
PCR Amplification (Phusion) cycles:	35

Experimental Set Up

Table 10: Concentration of samples used for the ovarhang PCR amplification.

Sample	Concentration [ng/μl]
pSB1C3-T7-Nuc (diluted from 288.6 ng/μl)	10
pSB1C3-T7-Nuc (diluted from 288.6 ng/μl)	48

Table 11: Volumes of components in PCR mix.

Component	Volume [μl]
10 μM Suffix_F	1
10 μM Nuc_R	1
5X Phusion GC Buffer	4
Phusion DNA Polymerase	0.2
2 mM dNTP mix	2
Template DNA	1
ddH_2O	10.8

Table 12: Thermocycling conditions, with two different annealing temperatures.

Step	Temperature [°C]	Time [sec]
Initial Denaturation	98	30
35 x	98	10
	57	30
	72	60
Final Extension	72	600
Hold	4	N/A

Results and Conclusions

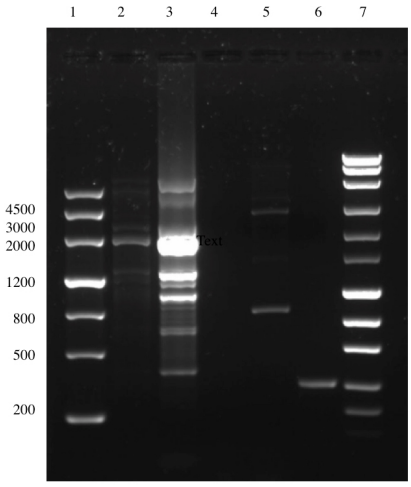

Figure 4: Overhang PCR of pSB1C3-T7-Nuc sixth attempt.(1) Ladder (2) Nuc with a template of 10 ng/μl (3) Nuc with a template of 48 ng/μl (4)-(6) Not of interest (7) Ladder

Discussion and Troubleshooting

Increasing the concentration of the nuclease construct template did not improve the results. The band for 10 ng/μl Nuc is at approximately 2000 bp, which is relatively far from the expected size of 2672 bp. This attempt had almost the same experimental conditions as the second attempt, except for the number of cycles. Thus confirming that the conclusion in the second attempt was wrong. The band seen in that attempt did not correspond to the expected size of 2672 bp.

Since the band seen in this attempt corresponds to a size smaller than the expected, future attempts will we done at a higher annealing temperature, with the other conditions kept.

5 Addition of BamHI restriction site downstream to the nuclease sequence and amplification of the whole plasmid, using overhang PCR - seventh attempt

Responsible

Oscar He

Protocols used

PCR Amplification (Phusion)

Modifications and comments to protocols

PCR Amplification (Phusion) primers:	Nuc_R (reverse) Suffix_F (forward)
PCR Amplification (Phusion) buffer:	GC Buffer
PCR Amplification (Phusion) annealing temperature:	60 °C

Experimental Set Up

Table 13: Concentration of samples used for the ovarhang PCR amplification.

Sample	Concentration [ng/μl]
pSB1C3-T7-Nuc (diluted from 288.6 ng/μl)	10
pSB1C3-T7-Nuc (diluted from 288.6 ng/μl)	45

Table 14: Volumes of components in PCR mix.

Component	Volume [μl]
10 μM Suffix_F	1
10 μM Nuc_R	1
5X Phusion GC Buffer	4
Phusion DNA Polymerase	0.2
2 mM dNTP mix	2
Template DNA	1
ddH_2O	10.8

Table 15: Thermocycling conditions, with two different annealing temperatures.

Step	Temperature [°C]	Time [sec]
Initial Denaturation	98	30
35 x	98	10
	60	30
	72	60
Final Extension	72	600
Hold	4	N/A

Results and Conclusions

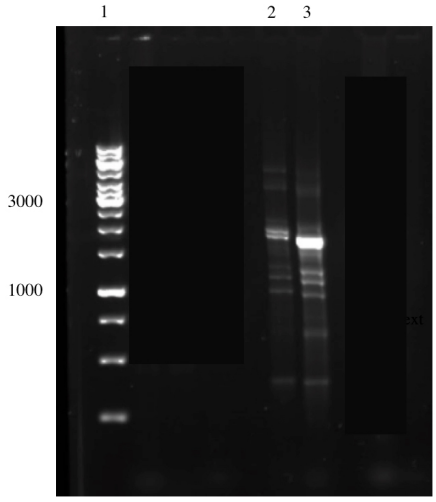

Figure 5: Overhang PCR of pSB1C3-T7-Nuc seventh attempt.(1) Ladder (2) Nuc with a template of 48 ng/μl (3) Nuc with a template of 10 ng/μl (4)-(6) Not of interest (7) Ladder

Discussion and Troubleshooting

The PCR run resulted in more contaminations and another try will be performed with an even higher annealing temperature. Also, DMSO will be added as one of two duplicates. This in order to eliminate any suspicions regarding difficulties in denaturing the template plasmid.

6 Addition of BamHI restriction site downstream to the nuclease sequence and amplification of the whole plasmid, using overhang PCR - eighth attempt

Responsible

Oscar He

Protocols used

PCR Amplification (Phusion)

Modifications and comments to protocols

PCR Amplification (Phusion) primers:	Nuc_R (reverse) Suffix_F (forward)
PCR Amplification (Phusion) buffer:	GC Buffer
PCR Amplification (Phusion) annealing temperature:	62 °C

Experimental Set Up

Table 16: Concentration of samples used for the overhang PCR amplification.

Sample	Concentration [ng/μl]
pSB1C3-T7-Nuc (diluted from 288.6 ng/μl)	45

Table 17: Volumes of components in PCR mix (without DMSO).

Component	Volume [μl]
10 μM Suffix_F	1
10 μM Nuc_R	1
5X Phusion GC Buffer	4
Phusion DNA Polymerase	0.2
2 mM dNTP mix	2
Template DNA	1
ddH_2O	10.8

Table 18: Volumes of components in PCR mix (with DMSO).

Component	Volume [μl]
10 μM Suffix_F	1
10 μM Nuc_R	1
5X Phusion GC Buffer	4
Phusion DNA Polymerase	0.2
2 mM dNTP mix	2
Template DNA	1
DMSO	0.6
ddH_2O	10.2

Table 19: Thermocycling conditions, with two different annealing temperatures.

Step	Temperature [°C]	Time [sec]
Initial Denaturation	98	30
35 x	98	10
	62	30
	72	60
Final Extension	72	600
Hold	4	N/A

Results and Conclusions

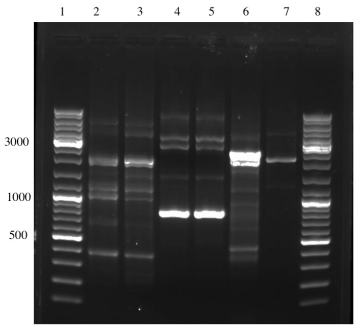

Figure 6: Overhang PCR of pSB1C3-T7-Nuc eighth attempt.(1) Ladder (2) Nuc without DMSO (3) Nuc without DMSO (4)-(7) Not of interest (8) Ladder

Discussion and Troubleshooting

The number of smaller sized contamination has not been changed significantly compared to the previous attempt. Therefore, the annealing temperature will be increased even more. Increasing the extension time will also be an option. The sample with added DMSO seems to have less contamination and more product of a size closer to the expected size of 2672 bp. In future attempts, DMSO will be added.

7 Kirby-Bauer test of expressed Nuclease

Responsible

Ellinor Lindholm

Protocols used

Kirby-Bauer test

Modifications and comments to protocols

Protein/antibiotic samples:	Kanamycin	50 mg/ml (positive control)
	Nuc 2.1.2 GS BL21(DE3) 3	no IPTG (negative control)
	Nuc 2.1.2 GS BL21(DE3) 3	0.5 mM IPTG
	Nuc 2.1.2 GS BL21(DE3) 3	1.0 mM IPTG
Incubation time	16 h	
Volume of TOB1 cells on McConkey plates	135 μl	

Experimental Setup

Two McConkey agar plates were plated with 135 μl Tob1 cell culture. Four whatman papers were soaked in 400 μl of each sample in Table 7 separately and placed in the Tob1 plated dishes. The plates were incubated in 37°C overnight.

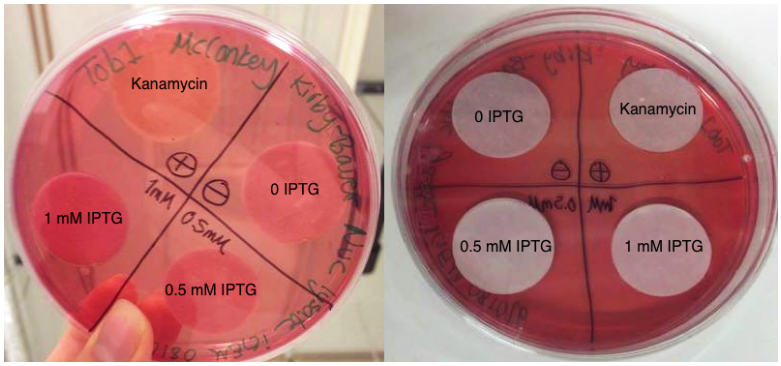

Figure 7: Figure showing the MacConkey plates with prepared and placed Whatman papers before overnight incubation. The figure shows the same plate taken from top and bottom.

Results and Conclusions

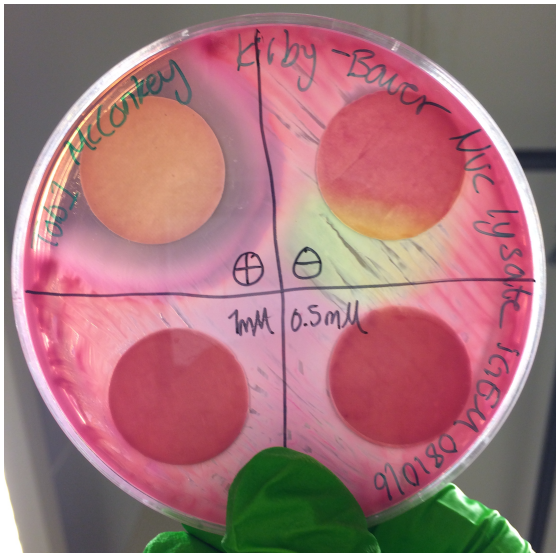

Figure 8: Result from the Kirby-Bauer test on the first plate.

Figure 9: Result from the Kirby-Bauer test on the second plate.

Discussion and Troubleshooting

The same cell lysates were used for this attempt. However, it was unsuccessful. There were no signs of impeded growth around the nuclease impregnated Whatman papers. There are three possible explanations to this result; i) the nuclease did not have a bactericidal effect on the TOB1 E. coli strain, ii) the concentration of nuclease in the cell lysate was too low or iii) the plasmid construct present in the BL21(DE3) cells used for expressing was not correct or had been unintentionally altered.

8 Addition of BamHI restriction site downstream to the nuclease sequence and amplification of the whole plasmid, using overhang PCR - ninth attempt

Responsible

Oscar He

Protocols used

PCR Amplification (Phusion)

Modifications and comments to protocols

PCR Amplification (Phusion) primers:	Nuc_R (reverse) Suffix_F (forward)
PCR Amplification (Phusion) buffer:	GC Buffer
PCR Amplification (Phusion) annealing temperature:	64 °C

Experimental Set Up

Table 20: Concentration of samples used for the overhang PCR amplification.

Sample	Concentration [ng/μl]
pSB1C3-T7-Nuc (diluted from 288.6 ng/μl)	45

Table 21: Volumes of components in PCR mix (with DMSO).

Component	Volume [μl]
10 μM Suffix_F	1
10 μM Nuc_R	1
5X Phusion GC Buffer	4
Phusion DNA Polymerase	0.2
2 mM dNTP mix	2
Template DNA	1
DMSO	0.6
ddH_2O	10.2

Table 22: Thermocycling conditions, with two different annealing temperatures.

Step	Temperature [°C]	Time [sec]
Initial Denaturation	98	30
35 x	98	10
	64	30
	72	60
Final Extension	72	600
Hold	4	N/A

Results and Conclusions

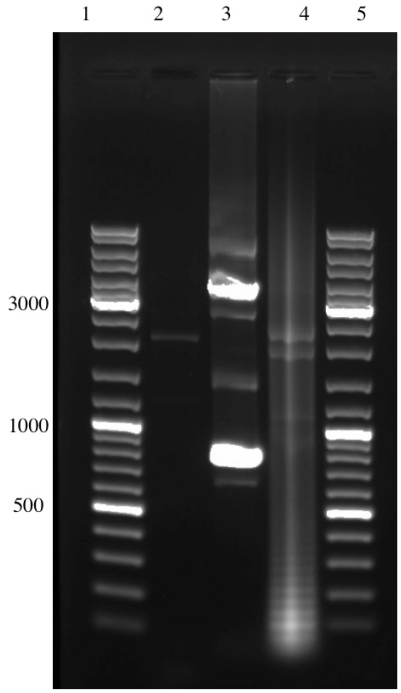

Figure 10: Overhang PCR of pSB1C3-T7-Nuc ninth attempt.(1) Ladder (2) Nuc with DMSO (3)-(4) Not of interest (5) Ladder

Discussion and Troubleshooting

The result show a single band without contaminations. But the band is at a smaller size than expected (2672 bp). For the next attempt, the annealing temperature will be increased even more.